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(54) Title: SARCOCYSTINE ISOLATION			
(57) Abstract <p>The present invention relates to a method of isolating a sarcocystine toxin from a mammal including the steps of: (a) dialysing blood serum from a mammal contaminated with a sarcocystine parasite against an aqueous salt solution; and (b) drying the dialysed solution to isolate purified sarcocystine toxin. The present invention also relates to the sarcocystine toxin when isolated by this method.</p>			

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SARCOCYSTINE ISOLATION

The present invention relates to a method for the isolation of the toxin sarcocystine
10 which is produced by a parasite of the *Sarcocystis* genus of protozoa.

More particularly, the present invention relates to a step by step procedural technique for isolating the toxin produced by a parasite of the *Sarcocystis* genus. In particular, material, preferably heart muscle, from an Intermediate Host (IH) with
15 *Sarcocystis* cysts is used to contaminate a Final Host (FH). After the development of the disease in the Final Host, a percentage of its blood is extracted and by means of a centrifugation process the serum is separated, then dialysed, preferably by passing through a sterile Physiological Saline Solution (PSS), and it is finally held for storage at a specific temperature.

20

One of the current known methods for isolating the toxin includes developing the toxin in macrocysts in sheep contaminated with the parasite. According to this method, the macrocysts which are formed in the oesophagus of the sheep can reach a visible size and contain relatively high quantities of toxin. Such a method entails the inconvenience that the
25 animal must be sacrificed to obtain a few millilitres of pure toxin. By contrast the animal is kept alive and in good health according to the method of the present invention, so becoming a good toxin donor from which a substantial volume thereof may be extracted regularly for a long time.

30

In an alternative known method, cell cultures may be utilised as supports for in vitro cultures of *Sarcocystis*, which allows for a totally-controlled environment to be maintained. The inconvenience arises from the fact that the parasite only begins to produce

the toxin 30 or more days after inoculation and then production ends shortly thereafter. Maintenance of these cultures for a long time becomes expensive and they yield a supernatant compound as complex as the animal's blood serum. This latter problem can be overcome using a purification procedure of the present invention.

5

The evidence of the infection with the parasite, known as sarcocystis in man, is usually accidental finding in the course of histopathological examinations. Protozoa of the Sarcocystis genus behave like an enzoonosis, and it has been reported all over the world that they are a causal agent of many pathologies in man. This parasite mainly affects lower 10 strata people with deficient nutrition although any person may be susceptible to it.

Protozoa of the Sarcocystis genus were reported for the first time in 1843 when a researcher by the name of Miescher found tubules in the skeletal muscle of a house mouse. Doctors Rommel and Heidorn in 1972 found that the protozoa's life cycle was 15 heterogeneous, with the asexual phase in the prey, the intermediate host, and the sexual phase in the pillager, the final host. At present, 122 species of the protozoa have been identified and in 56 of these two hosts are known. From the zoonosis point of view, those developing the asexual phase in humans are interesting, the final hosts of which are unknown to date, and the two which develop the sexual phase in humans which are known.

20

Considering all scientific knowledge at the world level concerning multiple sclerosis, lateral and amyotrophic sclerosis and the syndrome of chronic fatigue, there is no report of any link between a sarcocystis-like protozoa and these diseases. According to a review of the literature in the past months, we have found that multiple sclerosis is 25 considered to be closely linked to alterations in the immune system, mainly with alterations of the Tcd 4 lymphocytes.

Notwithstanding what has been expressed previously we have found that infection due to Sarcocystis gives rise to symptoms such as muscle spasms, intermittent diarrhoea 30 and chronic fatigue, even to multiple sclerosis. In our research, it has been determined that the pathogeny of this organism is caused by the toxin from Sarcocystis genus. It is desirable for this toxin to be isolated so as to be inactivated but in such a way that it

preserves its antigenic capacity, in order to obtain a specific immune-induced response from the part of the molecule immunologically responsive to a vaccine based on this toxin.

The present invention provides a method for isolating protozoa sarcocystine, i.e.,
5 the toxin of the *Sarcocystis* protozoa. This toxin may then be used in the production of toxides or vaccines and also in the production of specific antibodies against the toxin, as described and claimed in our co-pending British Patent Application No. 9603303.0.

In accordance with the present invention there is provided a method of isolating a
10 sarcocystine toxin from a mammal including the steps of:

- (a) dialysing blood serum from a mammal contaminated with the sarcocystine parasite against an aqueous salt solution; and
- (b) drying the dialysed solution to isolate purified sarcocystine toxin.

15 It is preferred that a mammal be used as a FH, e.g. a carnivore such as a canine animal. When a FH is used, it is also preferred that the mammal is infected with *Sarcocystis* parasite by muscle, and especially cardiac muscle, containing *Sarcocystis* cysts. It is particularly preferred that the dialysis solution consists of about 9 gm per litre of sodium chloride, although any salt solution of equivalent osmotic pressure can also be
20 used.

Preferably a mammal is used as an IH, wherein the IH is a herbivore, and wherein the IH is infected with sporozoites from the stools of the FH. Desirably the contaminated mammal has an absolute lymphocyte count at least 20% higher than its pre-contamination
25 count.

The invention also relates to the determination of the optimum time for bleeding the donor as explained herein below. This involves the use of the toxicogenic characteristics of sarcocystine. By using the technique of the present invention, it is possible to determine a
30 98.4% specificity (which means that examination of 100 patients positive to the toxin shows that 98 of them are detected by the test) and 99.00% sensitivity (which means that, upon examination of 100 patients negative to the toxin, 99 of them are detected by the test),

and it may be confirmed through biological titration. The toxin can be titrated in biological tests with a specificity of 99.80% (which means that upon examining 100 patients positive to the toxin, 100 of them are detected by the test), and a sensitivity of 99.99% (which means that upon examining 100 patients negative to the toxin, 100 of them are detected by the test).

The present invention also relates to the desiccation of the toxin isolated by dialysis in order to stabilise it for storage purposes.

In a preferred technique, a FH (eg, a canine animal), of which the absolute number of pre-infection lymphocytes is known, is contaminated with heart muscle of an IH (eg, a herbivore such as a bovine animal) containing *Sarcocystis* cysts. It is preferred that the heart muscle does not contain any other aetiological agent. Following this contamination of the FH, it is expected that such FH will develop sarcocystosis. The absolute lymphocyte count of the FH is an indication of when the next step in the procedure can occur. Once the lymphocyte count reaches a minimum of 20% above the pre-contamination count, biological titration of the toxin, as described herein below, can be applied to obtain a minimum titre of 3 units. At this point blood may be obtained from the FH. This is likely to occur between 50 and 80 days after contamination. The absolute lymphocyte count of the FH is an indicator of the toxin concentration due to the mitogenic capacity of sarcocystine. Sarcocystine is highly specific with respect to lymphocytes B, a feature not found in other substances from the blood of a mammal.

The FH is bled to obtain the required blood which is collected in a siliconed 500 millilitre sterile Erlenmeyer flask without anticoagulant. The serum is then separated at 800g for 20 minutes in sterile centrifuge tubes in order to eliminate the corpuscular blood components. The presence of a small degree of haemolysis is acceptable.

The serum is then dialysed against a sterile PSS composed of about 9 grams of sodium chloride in 100 cubic centimetres of distilled water for 36 to 48 hours, keeping the temperature between 18 and 25°C and shaking continuously.

At this point in the process, the toxin is isolated in the PSS with a minimum purity of 80%. Due to the size of the toxin molecule which is being isolated, temperature and dialysis time, it is possible to eliminate all those molecules which may cause alterations in the toxin usage.

5

The dialysed solution is then dried at a temperature of 37°C in order to remove the water and obtain the toxin concentrate. The dried toxin is then stored in sterile amber flasks and they are kept at room temperature in a dry place.

10

The remaining contaminants located in the desiccated portion of the dialysis are glucose and some materials, none of which affect the toxicogenic, physical and/or biochemical characteristics of sarcocystine.

15

This procedure results in the isolation of a molecule behaving like a histamine which must be examined in order to determine the toxicogenic titre which is found. It is important to make this titration since epitopes, i.e. the part of the molecule which is toxicologically active, is the same part of the molecule which is immunologically responsive to both humoral and cellular defences. Thus, a strain of the parasite which has proven production of very toxicogenic metabolites must be selected.

20

To select such a strain, the IH from which the strain is to be obtained must come from an area where the chronic sarcosporidiosis is frequent and with very severe symptoms of the chronic disease demonstrated by between 10,000 and 30,000 bradizooids per gram of wide dorsal muscle. It must also be verified through serological tests that the animal does not suffer from any other disease.

When the muscle which will be used to contaminate the FH is administered, sterile technique should be used. Utilisation of the cardiac muscle is recommended.

30

With this selected muscle, 4 to 10 FH young adults can be contaminated. These FH must show general vascular lesions and intermittent diarrhoea and that lymphocyte counts must exceed a minimum of 20% above the pre-contamination count.

Once these steps have been carried out and the titration of the toxin (as described below) shows the titre to be greater than or equal to 4 units, the conditions are suitable for the collection and purification of the toxin.

5 When high toxin volumes are required, the procedure should be applied to larger size mammals (eg, equine and bovine animals) with slight variations. A stool sample of previously contaminated FH, which has developed the whole picture described above, is taken and the sporozoites contained in the sample are purified. This is done by dissolving the stool sample in over-saturated glucose solution at the proportion of 1 volume of stool
10 per 3 volumes of over-saturated solution. The uppermost part of the preparation is collected, diluted in PSS and centrifuged at 300g for 10 minutes. The supernatant is discarded and the bottom residue is re-suspended in PSS to perform the toxin count.

15 The IH is contaminated with the parasite so prepared at a dosage of 700 sporozoites per kilogram. After 90 days, the IH is found in the chronic phase of the disease with high toxin contents in its blood, which makes it useful for extraction of the toxin. In order to confirm the concentration of the toxin, the same techniques as described above may apply to the FH, ie, lymphocyte count and/or biological titration of the toxin.

20 Between 40 and 60 days after contamination, the IH may show some symptoms of the severe disease, which may be treated symptomatically but no medicines for killing the Sarcocystis are to be used.

Titration Procedure

25 Three groups of 5 to 10 20-gram mice are selected. The toxin is diluted with PSS at two known concentrations, between 0.5 and 1 mg per ml. The first group of mice are orally administered with 0.2 ml of the toxin at dilution 1, the second group of mice are orally administered with 0.2 ml of the toxin at dilution 2, and the third group is used as a control and receives 0.2 ml of PSS without the toxin.

Eighteen hours later blood is obtained from all mice to make a total count of lymphocytes. They are then sacrificed to perform a necropsy.

In the event that the administration of the toxin has been successful, the mice that 5 received the toxin must present haemoperitoneum among other haemorrhages, while the control animals do not present any haemorrhage at all. Furthermore, the average of the total counts of lymphocytes in the inoculated animals will be higher than the average of the total counts of lymphocytes of the control animals.

10 An estimate of the toxin units ("U") is calculated according to the following formula:

$$\text{U/mg} = \frac{(\bar{Y}_{\text{Iri}} - \bar{Y}_{\text{Imi}}) \cdot 50}{\bar{Y}_{\text{Imi}}} \cdot d$$

Where: \bar{Y}_{Iri} = The average of the total lymphocyte counts in the 15 inoculated animals;

\bar{Y}_{Imi} = The average of the total lymphocyte counts in the non-inoculated animals;

d = Dilution factor in which the toxin was prepared.

20 A *Sarcocystis* type toxin unit is the amount of this toxin needed to increase by 10% the average of the total lymphocyte count of the contaminated mice as compared to the non-contaminated mice.

25 It will also be understood that the term "comprises" or its grammatical variants as used herein is equivalent to the term "includes" and is not to be taken as excluding the presence of other elements or features.

CLAIMS

1. A method of isolating a sarcocystine toxin from a mammal including the steps of:
 - (a) dialysing blood serum from a mammal contaminated with the sarcocystine parasite against an aqueous salt solution; and
 - 5 (b) drying the dialysed solution to isolate purified sarcocystine toxin.
2. A method as claimed in claim 1 wherein the contaminated mammal is a Final Host and is a carnivore.
- 10 3. A method as claimed in claim 2 wherein a mammal is used as an Intermediate Host.
4. A method as claimed in claim 3 wherein the Intermediate Host is a herbivore.
- 15 5. A method as claimed in claim 3 or claim 4 wherein the Intermediate Host is infected with sporozoites from the stools of the Final Host.
6. A method as claimed in any one of claims 1 to 5 wherein the contaminated mammal is contaminated with muscle containing *Sarcocystis* cysts.
- 20 7. A method as claimed in any one of claims 1 to 6 wherein the contaminated mammal has an absolute lymphocyte count at least 20% higher than its pre-contamination count.
8. A method as claimed in any one of claims 1 to 7 wherein the dialysis solution 25 consists of about 9 grams per litre of sodium chloride.
9. A method as claimed in any one of claims 1 to 8 wherein the dialysis step is carried out for about 36 to about 48 hours.
- 30 10. A method as claimed in any one of claims 1 to 9 wherein the dialysis step is carried out at a temperature between about 18 and about 25°C.

11. A method as claimed in claim 1 substantially as hereinbefore described.
12. Sarcocystine toxin when isolated by a method as claimed in any one of the
5 preceding claims.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00445

A. CLASSIFICATION OF SUBJECT MATTER		
Int Cl ⁶ : C07K 002/00; C07G 017/00; A61K 39/002		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) Keywords - see electronic database box below		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched See electronic database box below		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, JAPIO, MEDLINE, TOXLINE, CHEM ABS Keywords: Sarcocystin?, Sarcocystis and toxin?		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 600396 (UNIVERSITY TECHNOLOGIES INTERNATIONAL INC) 8 June 1994 see entire document	
A	Journal of the Egyptian Medical Association volume 52, no. 11, 1989 EI AKKAD et al., "on some pharmacological and toxicological effects of a protozoan toxin 'sarcocystin'"	
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TITLE: Isolating sarcocystine toxin from mammal - used for preparation of toxides and vaccines

INVENTOR: AZUMENDI, J L

PATENT-ASSIGNEE: AZUMENDI J L (AZUMI), SARCO RES CORP LTD (SARCN)

PRIORITY-DATA: 1996GB-0003304 (February 16, 1996), 1996AU-0043334 (February 5, 1996)

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<input type="checkbox"/> WO 9729120 A1	August 14, 1997	E	012	C07K002/00
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AU 9663492A	July 16, 1996	1996AU-0063492	
AU 9663492A		WO 9729120	Based on
AU 9643334A	February 5, 1996	1996AU-0043334	

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ABSTRACTED-PUB-NO: WO 9729120A

BASIC-ABSTRACT:

Isolating a sarcocystine toxin from a mammal comprises: (a) dialysing blood serum from a mammal contaminated with the sarcocystine parasite against an aqueous salt solution, and (b) drying the dialysed solution to isolate purified sarcocystine toxin. Also claimed is sarcocystine toxin isolated as above.

USE - The toxin can be used for the preparation of toxides and vaccines against protozoa of Sarcocystis genus. This parasite mainly affects lower strata people with deficient nutrition although any person may be susceptible to it. The protozoa may cause symptoms such as muscle spasms, intermittent diarrhoea and chronic fatigue and even multiple sclerosis.

ADVANTAGE - The animal can be kept alive and in good health and so can be used as a toxin donor from which a substantial volume of toxin may be extracted regularly for a long time.

ABSTRACTED-PUB-NO: WO 9729120A

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